# Lactic acid bacteria

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# Lactic acid bacteria: the bugs of the new millennium

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Lactic acid bacteria (LABs) are widely used in the manufacturing of fermented food and are among the best-studied microorganisms. Detailed knowledge of a number of physiological traits has opened new potential applications for these organisms in the food industry, while other traits might be beneficial for human health. Important new developments have been made in the research of LABs in the areas of multidrug resistance, bacteriocins and quorum sensing, osmoregulation, proteolysis, autolysins and bacteriophages. Recently, progress has been made in the construction of food-grade genetically modified LABs.

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**Abbreviations**

| | |
|---|---|
| **ABC** | ATP-binding cassette |
| **GMO** | genetically modified organism |
| **LABs** | lactic acid bacteria |
| **MDR** | multidrug resistance system |

## Introduction

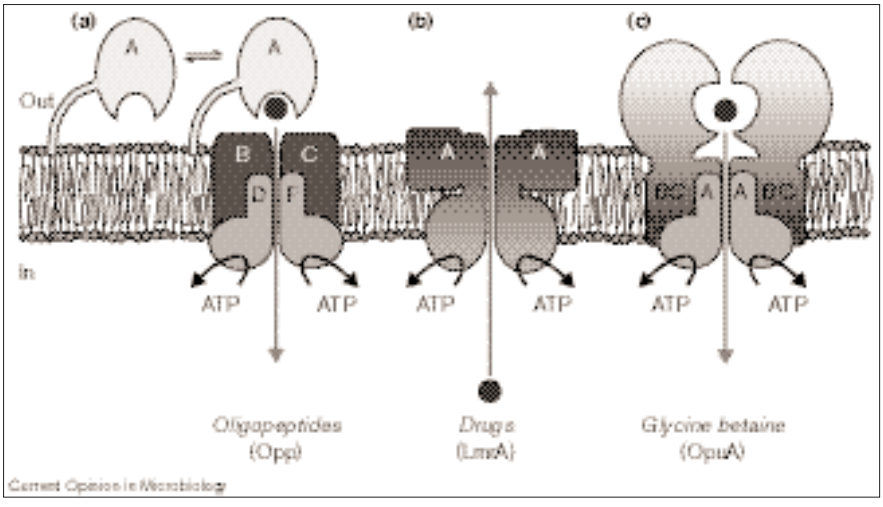
Lactic acid bacteria (LABs) belong to a group of Gram-positive anaerobic bacteria that excrete lactic acid as their main fermentation product into the culture medium. LABs were among the first organisms to be used in food manufacturing. Today LABs play crucial roles in the manufacturing of fermented milk products, vegetables and meat, as well as in the processing of other products such as wine. In order to understand and especially to manipulate the roles of these LABs in these fermentation processes, LABs have been studied extensively and are now among the best-characterised microorganisms with respect to their genetics, physiology and applications. The relative simplicity of LABs makes them excellent candidates for complete analysis of the metabolic pathways in the near future. Currently the genomes of several LABs are being sequenced and the first completely sequenced genome of the LAB *Lactococcus lactis* IL1403 has recently been presented [1••].

The extensive knowledge gained of LABs has opened new possibilities for their application. Tailor-made LABs with desired physiological traits can be constructed and can be applied to optimise the food manufacturing processes or to manipulate the organoleptic properties (i.e. the overall flavour and texture) of the products [2••]. In this review we concentrate on a number of physiological traits of LABs that have received significant attention recently for the reason that they might lead to exciting new applications of LABs.

## Multidrug resistance

Since the introduction of antibiotics around 1940, many pathogens have developed antimicrobial drug-resistance mechanisms. Different mechanisms of resistance in clinical isolates have been identified, including alterations of drug targets, modification of the drugs and reduced access of the drug to the intracellular target. In many microorganisms the reduced access of a variety of drugs was found to be the result of active drug-efflux systems. These so-called multidrug resistance systems (MDRs) are mainly responsible for the intrinsic or acquired resistance of microorganisms to antimicrobial drugs.

**Figure 1**




ATP-binding cassette transporters in *Lactococcus lactis*. **(a)** The oligopeptide transport system (Opp) uptakes peptides that have been processed by PrtP. OppA binds the peptide and donates it to the translocator complex of Opp in the membrane. **(b)** The multidrug transporter LmrA functions as a homologous dimer. Antimicrobial drugs are picked up in the inner leaflet and extruded in a medium. **(c)** The glycine-betaine-uptake system is activated by hyperosmotic stress. The glycine-betaine-binding domain (OpuABC) is linked to the translocator domain and the ABC-binding cassette domain (OpuAA).

In the LAB *L. lactis*, two multidrug-resistance transporters were found to confer resistance to cationic lipophilic cytotoxic compounds [3]. One of these transporters belongs to the major facilitator family and is called LmrP, while the other MDR is an ATP-binding cassette (ABC)-transporter, termed LmrA (Figure 1). Interestingly, this LmrA is the first ABC-transporter found in bacteria to confer multidrug resistance. Both LmrP and LmrA can excrete a wide variety of cytotoxic compounds, including the cytotoxic agents used in treatment of cancer cells [4].

LmrP and LmrA have been genetically and functionally characterised. The proteins have been solubilised from the membrane, purified and functionally reconstituted in liposomes [5••]. LmrP catalyses the excretion of lipophilic cationic compounds in exchange with protons and is responsible for *L. lactis* resistance to a variety of antibiotics, in particular those belonging to the group of macrolides and tetracyclins (M Putman, HW van Veen, JE Degener, WN Konings, unpublished data).

The other multidrug transporter of *L. lactis*, LmrA, is half the size of the human MDR1 P-glycoprotein, which is a heterologous dimer. P-glycoprotein plays a crucial role in the resistance of cancer cells against chemotherapeutic agents. Surprisingly, LmrA has about 50% similarity with each half of P-glycoprotein and functions as an homologous dimer [6••,7]. These observations demonstrate that these types of MDRs have been extensively conserved all the way from bacteria to man [4]. LmrA is not only a structural but also a functional homolog of P-glycoprotein. LmrA extrudes the same substrates and is sensitive to the same modulators as P-glycoprotein [6••].

The mechanism of extrusion of cytotoxic compounds by LmrP and LmrA has been studied in whole cells, isolated membrane vesicles and proteoliposomes. These studies revealed that the lipophilic substrates intercalate rapidly in the outer leaflet of the membrane. Subsequently, the substrate flips over slowly to the inner leaflet from where it is picked up by LmrP or LmrA and extruded in a proton motive force or ATP-dependent process to the external medium [8]. This 'vacuum-cleaner' activity of LmrP and LmrA has subsequently been confirmed for P-glycoprotein [9].

Recently, the role of LmrA in the resistance of *L. lactis* to antibiotics has been studied. Expression of LmrA in *Escherichia coli* confers this organism resistant to 17 out of the 21 clinically most used antibiotics, including antibiotics belonging to the classes of aminoglycosides, cephalosporins, macrolides, penicillins, quinolones, streptogramins and tetracyclins [10]. Thus, LmrA has the broadest substrate specificity reported for MDRs. In another LAB *Lactobacillus brevis*, a homolog of LmrA was found that confers resistance to the iso-alpha acids that are present in hop in the natural brewing process (K Sakamoto, A Margolles, HW van Veen, WN Konings, unpublished data). This LmrA homolog is encoded by the gene *horA*, which is located on a resistance plasmid. In view of the role of LmrA and HorA in multidrug resistance, it appears that these transporters may contribute to the intrinsic-drug resistance of the organisms in which they are expressed. Since bacteria are prone to gene exchange via conjugative or mobilisable plasmids and transposons to enhance their survival in drug containing habitats, a role for LmrA and HorA in acquired-drug resistance of (pathogenic) bacteria needs to be considered.

So far, homologs of LmrA and HorA have been found in the genome sequences of *Bacillus subtilis* and the pathogenic bacteria *Staphylococcus aureus*, *E. coli*, *Helicobacter pylori*, *Haemophilus influenza* and *Mycoplasma genitalium* [11]. In view of the extremely broad substrate specificity of LmrA, the role of these homologous systems in the antibiotic resistance of these pathogenic organisms should be seriously considered.

## Bacteriocins and quorum sensing

Bacteriocins (i.e. peptide antibiotics that are primarily lethal to other strains and species of bacteria) are produced by almost all genera of LABs, and can be divided in two main classes: the lantibiotics, which are post-translationally modified peptides, and the linear antimicrobial peptides. Interesting features of these peptides, apart from their possible applications either as food preservatives or as a basis for food-grade genetic modification and expression systems, are that they can interact with various different biomacromolecules. The lantibiotic nisin, which is one of the most potent bacteriocins, is known to be able to interact with at least nine structurally different compounds (Table 1), depending on its stage of biosynthesis and on its location. First, this small peptide can interact with other proteins — for example, those involved in the immunity mechanisms or the NisK signal transducer involved in 'quorum sensing' [12••]. Second, nisin can also 'dock' on

**Table 1**

**Compounds with which the bacteriocin nisin and its precursor can directly interact.**

| Compound | Properties |
|---|---|
| **Nisin interactions** | |
| NisI/NisFEG | Immunity; membrane proteins |
| NisK | Sensor histidine kinase |
| Lipid II | Cell-wall precursor/docking molecule |
| Phospholipids | Pore formation |
| Nisin | Pore formation |
| **Precursor–nisin interactions** | |
| NisT | ABC transporter |
| NisP | Processing of leader nisin-peptide |
| NisB | Dehydration |
| NisC | Lanthionine-formation |

the membrane-localised cell-wall precursor lipid II, enabling efficient membrane binding and probably also enhancing pore-formation [13•]. Third, nisin forms short-lived pores in biological membranes by interactions with neighbouring nisin molecules and with surrounding phospholipids, thereby killing the target bacteria [14].

Nisin and various other (linear) antimicrobial or pheromone peptides are sensed by LABs by quorum-sensing systems that enhance bacteriocin production when a certain threshold concentration of bacteriocin is reached [15,16]. The signal transducer NisK which senses nisin can be engineered in such a way that it has inverted sensor properties (i.e. constitutive promoter activity in the absence of bacteriocin and down-regulation of transcription in the presence of bacteriocin) [17•]. The knowledge on quorum sensing of LABs by means of their bacteriocin (or inducer peptide) concentration has led to the development of highly efficient, versatile, broad-host range controlled gene expression systems [18], which allow high overproduction of desired proteins, even those that are intrinsically toxic for the producing cell.

In the case of linear unmodified bacteriocins, such as plantaricins, carnobacteriocins and sakacins, the induction of bacteriocin synthesis is more complex. Specific-inducing peptides or peptide pheromones are produced that lack antimicrobial activity, but these peptides serve to stimulate production of the bacteriocin(s) typically encoded in the same gene cluster [19–23]. Also in some of these cases inducible gene expression systems could be developed. Undoubtedly, the advanced understanding of signalling mechanisms and the mode of action of bacteriocins will lead to useful applications in such diverse fields as gene expression systems, fighting multidrug resistance by providing alternatives for classic antibiotics [13•] and food applications by serving as natural preservatives.

## Osmoregulation

Maintenance of cell turgor is a prerequisite for almost any form of life as it is critical for growth and provides the mechanical force for the expansion of the cell wall. Since changes in extracellular osmotic pressure have the same physicochemical effects on cells from all biological kingdoms, the responses to osmotic stress may be similar in all organisms [24••,25]. Generally, (micro)organisms respond to hyperosmotic stress by rapidly accumulating compatible solutes to prevent the loss of water and loss of turgor pressure. Physiological studies on osmoregulation have revealed that the amino acids, glutamate and proline, and the quaternary ammonium compounds, glycine betaine (plant origin) and carnitine (animal origin), are the most important compatible solutes used by bacteria from various genera to protect themselves against hyperosmotic stress. A range of studies indicates that in the LABs *Lactobacillus plantarum*, *L. lactis* and *Listeria monocytogenes* glycine betaine and carnitine are the preferred compatible solutes. These compounds are taken up via transport systems that are activated upon hyperosmotic stress, whereas they are rapidly excreted by channel-like activities upon osmotic downshock [24••,26,27,28•].

The molecular properties of the hyperosmotic-stress-activated glycine-betaine-uptake system of *L. lactis* have recently been established. The system is essential for growth under hyperosmotic conditions and is probably paradigmatic for many osmotic upshift-activated transporters in (lactic acid) bacteria. The uptake of glycine betaine in *L. lactis* is effected by a unique ABC transporter (OpuA) that is composed of two different polypeptides (Figure 1). One of the subunits, OpuABC, comprises the glycine-betaine-binding domain linked to the translocator domain and the other subunit, OpuAA, comprises the ABC domain (Figure 1) [28•,29,30•]. Since binding-protein-dependent transporters generally function with two translocator and two ABC domains, the implication of the structure of OpuA is that two glycine-betaine-binding domains are present per functional unit. Furthermore, this work shows that ligand-binding proteins in Gram-positive bacteria are not necessarily associated with the outer surface of the cytoplasmic membrane via a lipid modification, but that in some systems the binding protein is linked to the cell surface via the integral membrane protein domain. Database searches indicate that several not yet characterised systems share the same architecture as OpuA. A clear advantage of this architecture is that protein purification and membrane reconstitution is facilitated, which has so far hampered the functional analysis of binding-protein-dependent ABC transporters. The purified and membrane-reconstituted OpuA system is fully functional in sensing and responding to changes in medium osmolarity [28•]. Preliminary data indicate that osmotic activation of OpuA involves the sensing of changes in the physical properties of the membrane bilayer that are brought about by the changes in medium osmolarity.

Hyperosmotic conditions are frequently used to preserve food products. In general, rather high concentrations of salts or sugars are needed to prevent outgrowth of spoilage or pathogenic bacteria. By interfering with the osmoregulated transporters directly or the signal transduction components that affect their expression, one will be able to apply milder conservation methods and thus improve the quality (i.e. flavour, texture) of the product without compromising the safety of the consumer.

## Proteolysis

LABs are multiple amino acid auxotrophs that utilise exogenous proteins such as caseins as a source of amino acids. The first step in the hydrolysis of β-casein by *L. lactis* is its protein breakdown by an extracellular cell wall bound proteinase PrtP. The activity of PrtP results in 5–30 amino acids fragments of casein that can subsequently be taken up by the cells for internal hydrolysis by a variety of peptidases. The role of the individual proteinases and peptidases in the proteolysis process has been assessed by constructing strains in which the expression of one or more enzymes is

lowered or increased. Since all these constructs have been made with food grade vectors, they can be used in the milk fermentation processes and the products can be evaluated by taste panels. In this way genetically modified LABs have been obtained that can be used in the production of cheeses with different organoleptic properties [31•,32].

A crucial step in the proteolysis process is the uptake of the relatively large peptides that are released by PrtP. This uptake is catalysed by an ATP-driven and binding-protein-dependent oligopeptide transport system Opp (Figure 1). The uptake of peptides of up to 10 amino acids has been shown *in vivo* using a mutant that is deficient in five peptidases and monitoring of intracelullar peptide accumulation by mass spectrometry [33••]. Subsequent specificity and size-restriction studies have shown that peptide fragments up to at least 18 residues can be taken up via Opp [34]. Clearly, this oligopeptide transporter has a different peptide spectrum than the homologous systems of *E. coli* and *Salmonella typhimurium*, which have high binding affinities for tri- and tetrapeptides. In contrast, Opp of *L. lactis* has very low affinities for these small peptides, while it has high affinities for larger peptides. This is reflected by the $K_d$-values of the *L. lactis* binding protein (OppA), which are >1000, 2.2 and 0.8 μM for the homologous peptides SLSQS, SLSQSKVLP and SLSQSKVLPVPQ, respectively [35••]. Peptides with a relatively low affinity are taken up with a comparable rate as those that have a high affinity [33••], the uptake of the 'high affinity' peptides appears to be rate-determined by the donation of the peptide from OppA to the translocator complex of Opp in the membrane [35••]. Transport of the low affinity peptides is not limited by this donation step but rather by the binding of the peptide to OppA. The notion that peptide uptake from complex casein hydrolysates is not simply determined by the affinity of peptide binding to OppA is supported by studies of OppA specificity mutants [36]. The crucial role of Opp in the proteolysis process is evident from the observation that an Opp-negative strain is unable to accumulate and hydrolyse PrtP-generated peptides in milk. Manipulation of the substrate spectrum of OppA by mutagenis can lead to an altered use of the peptides that are released in milk by PrtP and can affect the flavour development during cheese ripening.

## Autolysins, cheese ripening and oral vaccination

Environmental conditions leading to bacteriolysis of LABs have long been known. Many strains of LABs autolyse when incubated under conditions of 'unbalanced growth', which results in an inhibition of further cell wall peptidoglycan biosynthesis. For *L. lactis* it has been shown that cellular lysis observed during stationary phase is caused by the action of its major muramidase, AcmA. An AcmA-negative strain did not autolyse and formed long chains of non-separated cells. AcmA, thus, appears to be a major enzyme in determining cheese flavour development as AcmA-induced autolysis liberates flavour-forming enzymes in the cheese matrix.

The AcmA-negative strain of *L. lactis* was recently used to investigate the mechanism behind bacteriocin-induced lysis often observed in lactococci. Although the wild-type strain lysed upon addition of a number of different bacteriocins, the AcmA-negative strain did not. Apparently, the depletion of energy caused by the increase of membrane permeability brought about by the bacteriocins terminated wall synthesis, while the autolysin continued to break down the cell wall (CM Martìnez-Cuesta, J Kok, E Herranz, C Peláez, T Requena, G Buist, unpublished data).

AcmA is a modular enzyme: the active domain is in the amino terminus, while the carboxyl terminus contains a stretch of 45 amino acid residues that is repeated three times. Recently, it was shown that this repeat domain is involved in binding of the enzyme to its substrate, the cell wall [37•]. Interestingly, the cell wall anchor domain has a broad specificity as is indicated by the binding of AcmA to cells of a large number of Gram-positive bacteria. Although AcmA is anchored to the cell wall through its carboxyl terminus, part of the protein is released into the culture medium. Various active but truncated forms of AcmA were identified that lacked one or more repeats. Using genetically labelled strains it was shown that the released autolysin was able to attach to and lyse an autolysin-negative strain of *L. lactis*. Indeed, it was recently shown that the amidase also disrupts chains of *Streptococcus thermophilus* [38]. Again, in an indirect way, AcmA could be of importance to the performance of mixed strain starters.

The autolysin AcmA is hydrolysed by the lactococcal extracellular serine proteinase (caseinase) PrtP (see above) [39•]. The extent of AcmA degradation is largely determined by the proteolytic specificity of the proteinase. Consequently, the proteinase has a dual role in determining flavour formation during milk fermentation for cheese production. On the one hand, PrtP determines the composition of the initial casein breakdown products, and thus, the availability of substrates for the peptidases. On the other hand, it is involved in the degradation of AcmA, and in this way modulates the extent of starter cell lysis and release of intracellular flavour determining enzymes, such as peptidases and amino acid convertases.

The ability of the repeat domain of AcmA to bind to bacterial cells when added to these cells from the outside has recently resulted in a entirely new and exciting possibility to use non-genetically modified LABs for oral vaccination [40••]. By hooking up the repeat domain to other proteins through genetic engineering techniques, foreign antigens were presented on the surface of *L. lactis* in order to elicit the production of protective antibodies at mucosal surfaces.

## LABs and bacteriophage

All LABs are prone to phage attack and phage are among the main causes of fermentation failure. Consequently, phages are of great economic significance, a notion that has resulted in a major and worldwide effort to defeat them.

Two principally different routes have been taken to do this. First, the dairy industry has improved fermentation process technology to prevent phage infection. Air filtration, direct vat inoculation, the use of closed vats and phage-resistant strains as well as starter culture rotation are all daily practice in modern-day milk fermentation operations. However, all these technological advances have not eliminated phage as a serious threat, partly because the bacteria themselves are the source of phage. Many strains of LABs carry within their own genome a copy of a phage chromosome. It may even be common to find several, intact and/or remnant, phage genomes in one bacterial chromosome [1••]. Although still a matter of debate, it seems that in at least some LAB species lytic phages may have evolved from temperate phage [41].

The second approach in the conquest of phages exploits the tremendous potential of molecular biology tools currently available to the researcher in this area. The advance of molecular genetic methodology applicable to LABs, together with large-scale nucleotide sequencing techniques, has especially led to a tremendous increase in our knowledge of LAB phages. The chromosomes of thirteen phages infecting LABs, both temperate and lytic species, have been completely sequenced, allowing a thorough understanding of their structural and functional organisation [42••]. One of the major conclusions that can be drawn from comparing the phage genomes is that they are all highly organised: functionally related genes are clustered, with the clusters forming modules encoding proteins with a specific biological function. This modular organisation is seen as highly important to phage evolution, as recombination between partially homologous phages or interactions of phages with the host genome could lead to phages with new properties. From comparing phage genome sequences in more detail, however, it is clear that the theory of module exchange is too simplified: the module of genetic exchange can be as small as a gene (fragment). LAB phage evolution seems to be the result of a complex of modular exchanges combined with other genetic alterations such as deletions, inversions, duplications and point mutations [43•].

The dynamic evolutionary properties of phages have led to a search for natural as well as knowledge-based novel strategies to bridle their proliferation. Currently, each step in the phage life cycle (adsorption, DNA injection and replication, phage assembly and host lysis) can be specifically blocked and a number of the mechanisms have already been exploited in LAB strain improvement programmes [44,45].

Bacterial phage/host interactions also remain of prime interest to the future (dairy) fermentation industry. Among LABs are several bacteria that are potentially probiotic (Greek for 'pro-life') that, when administered to man or animal, benificially affect the host by improving the properties of the indigenous microflora. As the trait of the LAB in question may be exhibited only by one strain, its large-scale cultivation for use in a probiotic food may be very sensitive to phage attack. It would seem of utmost importance to thwart, already in an early stage, the emergence of phage attacking such strains.

## Food-grade approaches employing genetically modified LABs

To optimally use the physiological and genetic properties of LABs described above, it is of great importance to develop safe, stable and (cost) effective food-grade genetic modification, selection and expression tools. A recent review [46•] describes state-of-the-art approaches to selection and modification methods and to (inducible) gene expression systems. The following criteria are important for developing sustainable food-grade systems employing genetically modified organisms (GMOs) (modified from [46•]).

1. The selection of safe hosts, which should be well characterised, stable and with a long history of safe use.

2. The use of hosts that are food-compatible and devoid of unwanted selection markers such as antibiotic resistance.

3. The use of food-grade modification tools and genetic elements, preferably derived from self-cloning.

4. To never produce any harmful compounds (assessment of side-effects of the genetic modification might be necessary).

5. To be suitable for large industrial scale applications or for direct use in food products.

To conform to these criteria, food-grade selection and modification systems have been developed for various LABs [46•]. Food-grade selection markers include those based on sugar utilisation (lactose, sucrose), auxothrophic markers [47], and those that confer resistance or immunity to certain food-grade compounds, such as against bacteriocins. Nice examples that meet most of the criteria mentioned above are provided by Platteeuw *et al.* [48], who developed a versatile set of cloning and expression vectors for application in self-cloning based on lactose selection with *lacF* as a marker and *lacA* as an inducible promoter. More recently a system for a food-grade multiple-copy integration of desired DNA-fragments for *L. lactis* was described [49] that employs lactococcal plasmids and a pediococcal selection marker. Various inducible gene expression systems (e.g. based on lactose-, nisin-, phage-, NaCl-responding or purine-regulated promoters) have been developed, which all have their unique advantages and drawbacks [50].

A first example of the use of one of these systems in a real cheese application was described by de Ruyter *et al.* [51]. The NICE (nisin-controlled expression) system was used for the controlled lysis of *L. lactis*, where induction can

conveniently take place through *in situ* induction in curd by inclusion of low quantities of a nisin-producing strain. Significant enhancement of cheese ripening could be achieved by this approach, which is currently under investigation. A completely different approach that circumvents the inclusion of live GMOs in food products was recently described. A system was developed for anchoring previously produced recombinant proteins to host LAB cells by use of anchoring domains of LAB proteins (e.g. the repeated anchoring domain of the major lactococcal autolysin AcmA) [52•]. In this way cell-surface display of hybrid proteins can be achieved, yielding cells that can be exploited as oral vaccines (see above), for enzyme- or whole-cell immobilisation, for protein delivery to various environments such as the human gastrointestinal-tract or food products. Last but not least, recent developments in high-throughput technologies, such as genome sequencing and DNA-arrays, enable functional genomics efforts, which support GMO analysis to assess possible undesired side-effects of the genetic modification, and thus providing concrete tools to eventually ensure the safety of GMOs used in food products [53].

## Conclusions

The extensive knowledge that has now been accumulated about the physiology and genetics of LABs has led to a detailed understanding of several traits of these relatively simple bacteria. This knowledge can be used for improved as well as new applications of these organisms in food manufacturing. In addition, several properties of LABs may turn out to have clinical relevance and a number of strains may have health beneficial effects. For a number of physiological properties found in bacteria LABs have been proven to be excellent organisms for advanced analysis. Moreover, LABs are increasingly used as model organisms for physiological and genetic studies especially of Gram-positive bacteria.

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